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REC'D 17 JUN 2005
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/IB 2002/005253	International filing date (*day/month/year*) 9 December 2002 (09.12.2002)	Priority Date (*day/month/year*)
International Patent Classification (IPC) or national classification and IPC IPC[7]: C12N 15/82		
Applicant AVESTHA GENGRAINE TECHNOLOGIES PVT. LTD.		

1. This international preliminary examination report has been prepared by this International Preliminary Examination Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 31 sheets.

3. This report contains indications relating to the following items:

- I. ☒ Basis of the opinion
- II. ☐ Priority
- III. ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV. ☐ Lack of unity of invention
- V. ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI. ☐ Certain documents cited
- VII. ☐ Certain defects in the international application
- VIII. ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09.07.2004	18 May 2005 (18.05.2005)
Name and mailing address of the IPEA/AT Austrian Patent Office Dresdner Straße 87 A-1200 Vienna Facsimile No. 1/53424/200	Authorized officer MOSSER R. Telephone No. 1/53424/437

Form PCT/IPEA/409 (cover sheet) (July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/IB 2002/005253

I. Basis of the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages _____, as originally filed
pages _____, filed with the demand
pages 1-18, filed with the letter of 9 July 2003 (09.07.2003).

☒ the claims:
pages _____, as originally filed
pages _____, as amended (together with any statement) under Article 19
pages _____, filed with the demand
pages 19 (claims 1-12), filed with the letter of 9 July 2003 (09.07.2003).

☒ the drawings:
pages _____, as originally filed
pages _____, filed with the demand
pages 1-12, filed with the letter of 9 July 2003 (09.07.2003).

☐ the sequence listing part of the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____.

☐ the claims, Nos. _____.

☐ the drawings, sheets/fig _____.

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as „originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-6, 8-12	YES
	Claims	7	NO
Inventive step (IS)	Claims	----	YES
	Claims	1-12	NO
Industrial applicability (IA)	Claims	1-12	YES
	Claims	----	NO

Citations and explanations (Rule 70.7)

For the establishment of this examination report the Written Reply for the "Written Opinion from International Preliminary Examination Authority" was regarded. The Written Reply is dated 05.05.2005 (date of fax).

The following documents have been cited in the international search report:
D1: EP 359617 A2 (PLANT GENETIC SYSTEMS) 21.03.1990
D2: J. Plant Physiol. 1999, Vol. 155, pages 431-438
D3: Plant Sci. 1999, Vol. 148, pages 131-138
D4: J. Exp. Bot. 1999, Vol. 50, No. 330, pages 71-78
D5: Plant Physio. 1993, Vol. 103, pages 1155-1163
D6: US 5538878 A (THOMAS et al.) 23.07.1996
D7: Annals of Botany, 1999, Vol. 84, pages 543-547
D8: Biotechnol. 1994, Vol. 12, pages 165-168
D9: Plant Physiol. 1995, Vol. 107, pages 737-750

D1 concerns stress tolerant plants comprising recombinant DNA encoding a targeting peptide fused to the superoxide dismutase (compare present claims 1 and 5). The dismutase may be a MnSOD from Nicotiana plumbaginifolia (compare present claim 1). Further, the recombinant DNA can contain at least parts of the pea 1,5 ribulose bisphosphate carboxylase gene (compare present claim 2). According to D1 promoters and terminators are used (compare present claims 3 and 4). In D1 as well as in the present application the transit peptide facilitates the transportation of the MnSOD to a cell organelle especially to a chloroplast (see present claim 6). Furthermore D1 reveals the testing of stress tolerance of transgenic plants such as Arabidopsis thaliana. Also present claims 9-11 concern stress factors. D1 does not concern rice plants. Consequently D1 does not interfere with novelty. However, this document reveals the relevant tools and relevant information which are also mentioned in the present claims 1-6 and 9-11.

The results of the model used in D1 can be extrapolated to the system of rice because methods for producing transgenic plants are usually carried out with cells or plants in

an early state. In both cases a manipulation of cells or plant in an early state is done. Arabidopsis thaliana is a model plant. It is clear that a person skilled in the art will also try to establish similar experiments with plants which play an important role in food industry.

Claims 7 and 8 teach that rice varieties should produce high levels of SOD. This subject-matter is obvious because also the plants mentioned in D1 have higher SOD levels. D1 does not concern food industry but it is generally known that increasing of shelf life of food plants will play an important role in food industry.
D2 concerns the proteins FeSOD and MnSOD of Lupinus angustifolius and Nicotiana tabacum. D2 pertains to proteins and not to transgenic methods. Therefore this document does not interfere with novelty and inventive step.
D3 concerns transgenic rice overexpressing superoxide dismutase. Thus, the subject-matter of claim 7 is not novel. Although this document indicates that the overexpression of yeast MnSOD in rice chloroplasts conferred resistance to salt stress, its effect was very limited. The methods of D3 may not be the same as the methods of present claims. But if a person skilled in the art will combine the genetic methods of D4 with the techniques from D3 for the manipulation of rice plants he will come to the features of the present claims 1-6 and 8-12.

The applicant argues in the Written Reply that different transformation is used in D3. However, the present claims do not reveal if protoplast transformation by electro-poration or biolistic transformation of embryonic calli is done. Therefore this argument is of minor relevance. Further the genetic methods of D4 are not considerably different: The abstract of D4 concerns MnSOD from N. plumbaginifolia, pea ribulose-1,5-bisphosphate carboxylase, a well established virus promoter etc. All these technical features are also essential for the claimed invention.

D5 also does not concern rice but – as already carried out for D1 – this article concerns MnSOD from Nicotiana plumbaginifolia and the use of a transit peptide, promoters and terminators.

Considering the applicant's argumentation in conjunction with D5 the examiner concurs that there are differences between the present application and D5. But present claims neither show details for the marker nor for the transformation method (Agrobacterium mediated or biolistic method). However, it must be considered that CvMV promoter and NOS terminator are generally known by plant molecular biologists.

Therefore also D5 anticipates the subject-matters of present claims.
Finally also D6-D9 concern transgenic plants with elevated levels of SOD. These documents reveal many molecular biological techniques which are carried out with especially tobacco plants. The use of vector systems comprising sequences for a SOD, transit peptide, promoters and terminators are clear from these documents. Several environmental stresses which cause oxidative stress such as high light intensity, air pollutants, extreme temperatures, ozone, drought and heavy metals are mentioned in D7 and the other cited documents. Thus, although D6-D9 do not concern rice, no inventive step can be seen for the subject-matters of present claims.
Industrial applicability is obvious for the subject-matters of all claims.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 4 cannot be dependent from claim 4. However, the claim 4 is clear because it is obvious that an expression system will comprise a promoter and terminator.

PCT/IB 2002/005253

09 JULY 2003

Rice Conferring Resistance to Environmental Stress By Targeting MnSOD to the Chloroplast

Field of the Invention

The invention relates to an Indica rice transgenic plant that produces high levels of superoxide dismutase (SOD) and to a method for producing the said transgenic plant. Transportation of the expression product of the gene may be targeted to a specific cell organelle, such as the chloroplast. The SOD transgenic Indica rice thereby provides the means for cultivating crops in areas where it would not otherwise grow normally on account of environmental stress conditions inclusive of high and low temperatures, drought and ultra violet light, is resistant to herbicides thereby resulting in an increase in yield and also improved crop quality.

Background

Environmental stress in broad sense is a restriction placed on living organisms by nature. The definition of environmental stress in plant science is a set of physical and chemical factors affecting the environment consequently disturbing plant growth. This stress could occur due to many reasons such, as variant temperatures be it a high or low, insufficient water supply, ultraviolet radiation and emission of pollutant gases. The study of environmental stress in plant life is significant on account of the fact that agricultural productivity has been greatly restricted by it world wide and the need to withstand this kind of environmental stress is a prerequisite for the plants that grow in such conditions.

Under stressful conditions, the stress factor or toxic molecules that are derived as a result of stress experienced by the plants, attack the more sensitive molecules i.e. the primary targets in cells to impair their functions. Cells are protected by the endogenous molecular systems that mitigate the stress. The damaged targets are recovered either by having them repaired or replaced via de novo biosynthesis. When the damage caused by stress to the primary targets is very intense, the cell cannot get over the damage and the metabolic function(s) operated by the target molecules are distorted. If the repair system in respect of the damaged molecules or the energy supply system is impaired, viz., the entire cellular metabolism disintegrates allowing for the propagation of damages, then there is a cascade of events leading to cell death.

Cells have the capability of surviving stressful conditions by sensing stress and adjusting their gene expression pattern to establish new metabolism, which adapt to the stress. This adaptive response is known as acclimatization and it takes place from few hours to several days in which time cells take on the stress by making use of pre existing protection systems until the new metabolism is established. The destiny of the cell is determined by the degree of available protection and the intensity and duration of the stress. The investigation on the cellular response in the early stages of environmental stress has revealed that the endogenous and exogenous factors determine the stress tolerance of a plant.

The production of reactive oxygen species in cells is an inevitable restriction on aerobic life and use is made of the oxidative atmosphere for yielding energy at a high efficiency. In so far as the

metabolism under non-stressful conditions is concerned, reactive oxygen species is always produced. The reactive oxygen species is produced in the cells for biosynthesis, cell defence, intra and intercellular signalling. Hence, reactive species of oxygen is both, indispensable as well as toxic to life.

It has been observed that reactive oxygen species plays crucial role in the impairment of cellular functions due to environmental stress viz., increase in the production of reactive oxygen species which further results in the production of oxidised target molecules under stressful conditions. This leads to decrease in the antioxidant levels in plants under stress. As a result of which an increased expression of the genes is seen in plants for anti-oxidative functions by stress. It has been reported that there is a positive co-relation between the scavenging capacity of the reactive oxygen species and tolerance towards stress and cross tolerance between oxidative stress and other stress.

Oxidative damage caused by reactive oxygen species can be induced by two principal mechanisms viz, an enhanced production of reactive oxygen species or by an inhibition of the scavenging systems for them. The damage proliferates production of a highly reactive hydroxyl radical and the subsequent reactions like bleaching of pigments and accumulation of oxidised lipids are apparent, these being the final symptoms of oxidative damage observed in dying cells.

Superoxide is commonly encountered mediate of oxygen reduction. It is extremely toxic to cells since it attacks unsaturated fatty acid components of membrane lipids thereby damaging the membrane structure. Aerobic cells detoxify super oxide by the action of super oxide dismutases, metal containing enzymes that convert the superoxide radical into hydrogen peroxide and molecular oxygen. The hydrogen peroxide later converted by catalase into water and molecular oxygen.

There are three types of super oxide dismutase (SOD), copper/zinc containing SOD(CuZnSOD), manganese containing SOD (MnSOD) and iron containing SOD (FeSOD). In prokaryotic organisms MnSOD is inducible under conditions of high oxygen concentration and by O@.

Reactive oxygen species is produced in almost every cell compartment in instances of normal metabolism (Halliwell & Gutteridge, 1989). The chloroplast containing pigments at high concentrations and evolving 0_2 under light is a major source of reactive species in plant cells under illumination (Asada & Takashahi, 1987). The chloroplast and the leaf tissue is regarded as a primary site of stress induced damage in plants under light. However there are numerous cases wherein the stimuli arise from extrachloroplastic sites, e.g., the ozone, which, penetrates into the leaf tissue and interacts with apoplastic components on account of which the apoplastic antioxidant capacity assumes significance. Biotic stress like bacterial infection and grazing arises from the periphery of the cells. In the case of water stress like drought and high saline content in the soil, stress stimuli are sensed primarily by roots as well as the leaves.

In an instance where the chlorophyll (Ch1) molecule at the photochemical reaction centre in the thylakoid membranes absorbs light energy, a high potential oxidative power i.e. a positive charge and a low potential reducing power, a negative charge is generated. On the oxidative side of the photosystem II (PSII) the oxidative terminus of the photosynthetic electron transport chain, water

is oxidised to 0_2. On the reducing side of photosystem I (PSI) the opposite terminus, the iron-sulphur protein ferredoxin (Fd) is reduced. The reduced Fd provides electrons for CO2 fixation and other reactions in the chloroplast. There are two potential production sites for reactive oxygen species, the reducing side of PSI and PSII.

The redox potential of the FeS centres at the terminus of PSI, ə 0.4 is low enough to reduce 02 univalently to produce superoxide radical (02ə). 02 + PSI reduced → 02ə + PSI (oxidised). The photoreduction of 02 to 02ə by PSI (Asada & Kiso, 1973b) inevitably occur and uses 10-20% of the photosynthetic electron flux even under conditions where C02 supply saturates (Asada & Takahashi, 1987). 02ə is disproportionate to H202 and 02 via catalysis by superoxide dismutase (SOD) which is contained in the stroma (Asada et al., 1973).

2 02ə + 2 H+ → H_2O_2 + O_2: These reactions account for most of the photoproduction of H_2O_2 in chloroplast (Mehler reaction; Mehler, 1951). H_2O_2 is produced via non-enzymic reduction of O_2ə with ascorbate (AsA) or glutathione (GSH). O_2ə + AH → H_2O_2 + A∀, where AH and A∀ represent either As A or GSH and its radical, respectively. Under normal physiological conditions this mechanism is neglected since the produced O_2ə is immediately disprorportioned with SOD, which resides near the production site of O_2ə (Ogawa et al., 1995).

02 photoproduced from H20 in PSI II is finally reduced to H20 in PSI, with catalysis by SOD and APX, to form a cycle of electron flow (water-water cycle; Asada et. Al, 1998). With regard to the produced reactive oxygen species scavenged in situ by the enzymes of the water cycle, the photoreduction of 02 to 02ə is not detrimental but indispensable in preventing photoinhibition of chloroplast by acting as a safety valve that dissipates excessive excitation energy as heat (Schreiber & Neubauer, 1980, Neubauer & Yamamoto, 1922, Osmond & Grace, 1995, Laisk & Edwards, 1998). Even at 1.1 $^{0/0,}$ C02 that saturates photoreduction of C02 in chloroplast, the electron flow to 02 prevents photoinhibition despite producing 02ə (Park et al., 1996). This efficient scavenging of 02ə and H202 is ensured by high molecular activities and intraorganellar microlocalisation of the water-water cycle enzymes (Asada et al., 1998). The chloroplastic flavoenzyme monodehydroascorbate reductase has been suggested to regulate the photoproduction rate of 02ə at PSI (Miyake et al., 1998).

H202 is also produced outside the chloroplast not only via the disproportionation of 02ə but also via the divalent reduction of 02 catalysed by various oxidases which catalyse divalent oxidation. H202 if provided with reductants and an appropriate catalyst, e.g. transition metal ions, quinones and Fd (Jacob & Heber, 1996), is reduced to form a highly toxic hydroxyl radical (HOψ) (Heber-Weiss reaction). H202 + AH → HOψ + OH ə + A. AsA, GSH and 02ə can be reductants for this reaction. As catalysts the FeS centres in PSI reaction complex (Sonoike, 1996b) and in Fd (Jacob & Heber, 1996) might produce HOψ in situ. Transition metal ions e.g. Fe, Cu and Mn, if released from metalloenzymes for some reasons are also effective catalysts. Cd from the environment also catalyses the Haber-Weiss reaction. HOψ production is implied in the oxidative stress caused by excess Fe in tobacco (Kampfenkel et al., 1995). HOψ can also be detected on the donor side of PS II which is impaired by UV-B (Hideg & Vass, 1996) although the source and the reaction to produce this radical is not yet known, as of now, HOψ is highly

oxidative (redox potential of HOψ/H20; +2.3 V) and oxidises organic molecules at the constant rate of 109 MD sD1 (Halliwell & Gutteridge, 1989) and is toxic.

At the other end of the electron transport chain, at the time when the charges separated at the Ch1 dimer at the reaction centre recombine, the triplet state of Ch1 (3Ch1) is formed and it reacts rapidly with ground state oxygen (302) to form a singlet oxygen (102). 3Ch1 + 302 → 1 Ch1 + 102. 102 is also produced via a similar photodynamic reaction with heme groups in proteins and with flavins through various reactions from 02D and H202 (Halliwell & Gutteridge, 1989). In PSI II reaction centre, 102 is produced when the primary acceptor quinone QA is fully reduced (Vass & Styring, 1993). The photoproduction of 102 in PSI II has been observed in vitro (Macpherson et al., 1993) and in vivo (Hideg et al., 1998). 102 is highly reactive with organic molecules and consequently, highly toxic as well. The oxidative potential generated in the PSII reaction centre required for the oxidation of water to oxygen is potentially toxic to the PSII complex itself and damages it as a probable event (Anderson et al., 1998). The oxidant is harnessed with a charge accumulation mechanism of the Mn cluster of water oxidase (Kok et al., 1970) so as not to release the possibly generated intermediates of water oxidation, HOψ, H202 and 02D. When water oxidase is destroyed on account of some reason or the other, such as UV-B or heat, the photogenerated oxidative power as P680+ or Tyrz+, may, oxidise the surrounding protein matrix or neighbouring molecules to inactivate PSII complex (donor-side-induced photoinhibition; Blubaugh et al., 1991, Aro et al, 1993). Further, reactive oxygen species that can be produced through photooxidation of water, may be released (Ananyev et al., 1992, Fine & Frasch, 1992, Hideg et al., 1994).

In additional chloroplastic compartments, the major production reaction for reactive oxygen species are not only the univalent reduction of 02 to 02D but the divalent reduction of 02 to H202. Peroxisomes contain divalent reaction oxidases and produce H202 in association with oxidative metabolisms like photorespiration and –oxidation of lipids. In C3 plants a substantial amount of H202 is produced and accompanies the photorespiration through the peroxisomal glycolate oxidase.
02 + glycolate → H202 + glycolate.

Acyl-CoA oxidase in peroxidase catalyses divalent oxidation of acyl-CoA to trans-2, 3-dehydroacyl-CoA by 02 in the beta-oxidation of lipids, producing H202. 02D is produced in mitochondria. In mammalian mitochondria, 02D production due to electron leakage from the electron transport to 02 accounts for 1-2% of total electron flux through the chain (Chance et al., 1979) and is increased several fold by the inhibitors of electron transport, uncouplers and other agents to disrupt mitochondrial functions (Richter & Schweizer, 1997). The production of 02D in submitochondrial particles from pea leaves has been demonstrated (Hernandez et al., 1993). Assuming that mitochondria is a major production site of 02D in non photosynthetic cells, it has not yet been elucidated as to whether the production of 02D in mitochondria has a physiologically positive significance as that in the chloroplast. O2D is also produced in peroxisome and plasma membrane. In plant peroxisome, O2D is produced via xanthine oxidase and at least three distinct NAD (P) H oxidases (del Rio, 1998). Peroxisomal 02D production is increased during senescence and the reactive oxygen species derived from it, decompose cellular components (Brennan & Frenkel, 1977, del Rio et al., 1998). Participation in the production of 02D of a mammalian like NADPH-oxidase on the plasma membrane in plant cells has been

established upon extracellular stimuli (Auh & Murphy, 1995, Allan & Fluhr, 1997) and during lignification (Ogawa et al., 1997).

Reactive oxygen species have their respective molecular properties and reactivities with biomolecules with scavenging mechanisms for both. O2D is generally known as a relatively stable or unreactive molecule among the reactive oxygen species. However the protonated form H02 (pKa = 4.8) is a much higher reactive. H02 can initiate lipid peroxidation but not 02D. Moreover H02 can pass across lipid bilayers but not 02D. In an aqueous solution, 02D spontaneously disproportionates to form H202 and 02.
02D + 02D + 2 H+ → H202 + 02
At a lower pH, the following reactions may occur:
02D + H02 + H + H202 + 02
H02 + H02 → H202 + 02
The second order rate constants for these reactions are <0.35 MD1 sD1, 1.02 x 107 MD1 sD1 and 8.60 x 105 MD1 sD1. Since the reaction constant is the largest apparent second order rate constant for the disproportionation of 02D, 5-105 MD1 sD1 at pH 7.0, thus decreases by 10 fold per each pH unit increase in the range over pH 5 (Bielski, 1978). 02D is a reductant of the transition metal ions in the Haber-Weiss reaction to produce HOψ from H202. 02D also propagates radical chain reaction especially in the presence of quinone. When quniones are univalently reduced to semiquinones (QHψ) with quinone reductases which abundantly occur in plant cells, parts of the QHψ reduces dioxygen to produce 02D, which oxidises the quinols that have been produced via the disproportination of QHψ to reproduce QHψ. This chain reaction is effectively terminated by SOD (Cadenas et al., 1992).

O2D is highly reactive with reduced sulfur compounds like thiols and FeS cluster. O2D oxidises thiols to the thiyl radicals at diffusion controlled rates (Asada & Kanematsu, 1976). The resulting thiyl radicals initiate radical chain reaction. 02D also oxidises the 4 Fe-4S) cluster of aconitase in mammalian mitochondria or in bacteria at the order of 106-107 MD1 sD1 to the inactive (3Fe-4S) form (Radi et al., 1998). The Fe2 + ion released as a consequence is a potent catalyst for Haber-Weiss reaction. In plant cells the major SOD isozymes are located in chloroplasts (MnSOD). The occurrence of CuZnSOD in the apoplast and nucleus has been confirmed by immunoelectron microscopy (Ogawa et al., 1995). The occurrence of SOD implies the in situ production of 02D. CuZnSOD and FeSOD are sensitive to H202. These SODS are the potential targets if the H202 scavenging systems do not operate properly.

H202 is a neutral, non radical molecule below pH 10and can diffuse across biomembranes like water. The function of H202 as a stress signal (Doke, 1997) is partly based on its intra and inter cellular diffusability. H202 is a relatively weak oxidant, the oxidative potential of H202/H20 pair is + 320 mV. However, metalloenzymes are in general sensitive targets of H202. Heme proteins can catalyse the Haber-Weiss reaction and can be degraded by the resulting HOψ (Puppo & Halliwell, 1998). Chloroplastic APX isozymes are inactivated by H202 in the absence of electron donors (Hossain & Asada, 1984) since compound I is irreversibly oxidised by H202 (Miyake & Asada, 1996). CuZnSOD is inactivated by H202 (Bray et al., 1974) through the reduction of Cu2 + ion at the reaction centre to Cu+ and the subsequent production of HOψ (Hodgson & Fridovich, 1975). CuZnSOD in isolated chloroplast of wheat leaves are inactivated by insufficient light probably due to photoproduced H202 (Casano et al., 1997). FeSOD is also

inactivated by H202 (Beyer & Fridovich, 1987). The inactivation of these enzymes have been observed in vitro at the _M to sub-mM range of H202, which can be reached in vivo as well if the H202 scavenging systems do not operate effectively. H202 oxidises thiols to the sulfenic acids which react withthiols to form disulfides. The reaction between H202 and cysteine is slow (the apparent second order rate constant, 1MD1, sD1) but on the surface of the proteins the reaction may be largely accelerated by the presence of basic residue like Lys and Arg which could be the neighbouring thiol groups 9Armstrong & Buchanan, 1978). H202 at micromolar concentrations in darkness inhibit C02 fixation in the chloroplast by 50% in 10 min (Kaiser, 1979) due to the oxidation of the active site thiols to the disulfide in the Calvin cycle enzymes; fructose-1; biphosphatase, NADP- glyceraldehydes-3-phosphate dehydrogenase and ribulose – 5 phosphate kinase. The activities of these inhibited enzymes are recovered by the reduction with reduced thioredoxin reversibly (Wolosiuk & Buchanan, 1977). However, if cessation of C02 continues under light, it will lead to excess light energy wherein the production of reactive oxygen species increases.

H202 is scavenged by two types of enzymes, catalase and peroxidase. The former scavenges H202 through the disproportionation of H202 to 02 and H20 corresponding to a turnover rate of about 107 minD1. (Scandalios et al., 1997). 2H202 –02 + 2 H20. Plants have several catalase isozymes, which are expressed in the regulated stage and tissue (Scandalios et al., 1997). Catalase is localised mainly in peroxisomes and responsible for scavenging the H202 produced in photorespiration and beta-oxidation of lipids. Catalase is a key antioxidant enzyme, a tetrameric heme containing enyme found in nearly all the aerobic organisms which converts hydrogen peroxide into water and molecular oxygen in plants and are primarily located in peroxisomes. Plant catalases are involved in the detoxification of active oxygen species which are generated during the course of photorespiration, the beta-oxidation of fatty acids or different environmental stresses (Scandalios, 1990).

It has been shown that induction of superoxide dismutase activity in plant cells has been correlated with development of increased tolerance to a variety of chemical compounds and physical stress. Environmental stress is known to decrease crop activity according to the severity and type of stress. Enhancing tolerance of crop plants to adverse effects imposed by non optimal growing conditions for improvement of crop management. There is hence, a substantial interest in the ability to increase the concentration of super oxide dismutase in a plant cell so as to provide for a plant which has increased tolerance to environmental stress.

Summary of the invention

The present invention necessitated a comparative study of the MnSOD gene expression at mRNA level during the abiotic stress in the seedlings of contrasting *indica* rice varieties, IR64 and RASI, and offers a simple yet powerful tool to monitor alterations in the gene expression and further, has proved the dramatic induction of MnSOD during stress conditions. Further, the superoxide radical triggers a specific molecule in each sub cellular compartment, which is capable of acting as a signal to induce nuclear gene encoding for the particular superoxide dismutase associated with that compartment.

Transgenic japonica rice *Oryza sativa* plants have been generated that over express *Nicotiana plumbaginicolia* L., MnSOD in the chloroplast. These transgenic plants containing chloroplast targeting peptide MnSOD gene are resistant and or tolerant to naturally occurring environmental stress conditions, during which highly reactive oxygen species in various compartments of the plant cells are produced. Thus, increasing the quality and potential yield of the transgenic rice plants.

In the present invention, we have generated transgenic *Japonica* rice plants by co-transforming three week old scutellum derived rice japonica callus using particle accelerator Biolistic PDS-1000/He with the plasmids, pGV2 and pILTAB122. The plasmid pGV2 carried the MnSOD cDNA (Bowler et al, 1989), cloned downstream of CVMV promoter and the chloroplast targeting peptide followed by the NOS terminator. The pILTAB122 carried the hygromycin B phosphotransferase downstream of 35S CVMV promoter, followed by the NOS terminator.

On transformation of three-week old rice scutellum derived calli with pGV2 and pILTAB122, 20 transgenic lines were obtained of which 11 were positive for hygromycin B phosphotransferase gene and MnSOD gene. The 11 transgenics obtained were used for physiological and biochemical assays. The transgene, MnSOD, has a chloroplast targeting peptide from the small sub-unit of rubisco (ssTP), therefore it was expected that the translated product of the transgenes in the chloroplasts. Immunolocalization studies that were carried out confirmed the presence of transgene in the chloroplast of transformed plants.

Various physiological and biochemical assays were done to show that the transgenic MnSOD performance is better than the performance of untransformed under stressed conditions. Out of the 11 transgenic lines that are positive for hygromycin B phosphotransferase gene and MnSOD gene, 2 lines i.e., Godavari 8 and Salween 2 showed higher levels of SOD activity and SOD protein content than the untransformed controls.

Conductance measurement that reflects oxygen-scavenging ability showed greater protection in case of transgenic Godavari 8 and Salween 2 than that of the controls on performing conductance assay. On doing chlorophyll fluorescence assay, transgenic lines showed healthier chloroplast even under methyl-viologen treatment when compared with controls. Further catalase levels were up regulated in the transgenic line Godavari that had higher SOD activity. Also, preliminary study for looking at cell viability under stress showed that the transgenic Godawari performed better among transgenics developed and among controls as well.

It is therefore the principle object of the present invention to develop engineered transgenic rice plants resistant to environmental stress which includes herbicide resistance, high and low temperature, ultra-violet light and drought.

Yet another object of the present invention is to optimize the use of genetically engineered over-expression of MnSOD targetted to chloroplast for resistance and or tolerant to environmental stress.

Still further object of the present invention is to correlate the over-expression of the MnSOD targetted to the chloroplast in the transgenic lines with the environmental stress induced MnSOD gene expression. The stress resistance in the transgenic lines is the consequence of scavenging of super-oxide radicals from the chloroplast by MnSOD in transgenic lines.

Said environmental stress resistant transgenic *japonica* rice plant is selected from a group of *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Zea mays* (Corn), *Glycine max* (Soybean) and the like.

Said environmental stress resistance and or tolerant transgenic *japonica* rice plant is *Oryza sativa* (rice) plant.

The present invention relates to a process for preparing environmental stress resistance and or tolerant transgenic system comprising of the folowing steps:

a. Cloning of 1.1 kb chloroplast targeted peptide MnSOD gene from pChltpMnSOD into pBSK SK- vector background.

b. Cloning of 1.1 kb chloroplast targeting peptide MnSOD gene by restrict digestion of pBSK SK- using kpnI and BamHI restriction enzyme into pLAU6 in between CvMv promoter and NOS terminator to obtain pGV2.

c. Cloning of hygromycin B phosphotransferase gene in between 35s CaMv promoter and NOS terminator of pMON1999 to obtain pILTAB122.

d. Co-bombarding of pGV2 and pILTAB122 constructs to generate transgenic plants containing chloroplast-targeting peptide MnSOD gene.

e. Molecular analysis such as PCR, southern blotting, northern blotting and western blotting of the transgenic lines containing chloroplast targeting peptide MnSOD gene.

f. Biochemical and physiological analysis of the transgenic lines such as super-oxide dismutase assay, immunolocalization assays, methyl-viologen tolerance leaf disc assay, chlorophyll fluroscence assay, assay for MDA by ESR, assay for catalase activity, cell viability and root growth assays.

Transgenic japonica rice Oryza sativa plants have been generated that over express Nicotiana plumbaginicolia L., MnSOD in the chloroplast. These transgenic plants containing chloroplast targeting peptide MnSOD gene are resistant and or tolerant to naturally occurring environmental stress conditions, during which highly reactive oxygen species in various compartments of the plant cells are produced. Thus, increasing the quality and potential yield of the transgenic rice plants.

In the present invention, we have generated transgenic *Japonica* rice plants by co-transforming three week old scutellum derived rice japonica callus using particle accelerator

Biolistic PDS-1000/He with the plasmids, pGV2 and pILTAB122. The plasmid pGV2 carried the MnSOD cDNA (Bowler et al, 1989), cloned downstream of CVMV promoter and the chloroplast targeting peptide followed by the NOS terminator. The pILTAB122 carried the hygromycin B phosphotransferase downstream of 35S CaMV promoter, followed by the NOS terminator.

20 putative transgenic lines were obtained as a result of co-transformation of scutellum derived rice calli using particle accelerator biolistic PDS-1000/He with pGV2 and pILTAB122 the presence of the gene from T_0 to T_4 generations have been proved by PCR, southern blotting, northern blotting and western blotting. Out of 20 putative transgenic T_0 lines, 17 lines were positive for hygromycin gene and 11 lines were postive for hygromycin and chloroplast targeting peptide MnSOD gene. The 11 lines that were positive for MnSOD and hygromycin gene were selected for biochemical and physiological experimentation. The product of native engineered protein has been assayed and immunolocalised to the Chloroplast. Out of the 144 lines from the T_1 generation 14 lines are selected for further analysis to carry out physiological experiments for SOD and Oxidative stress. These include herbicide resistance, high and low temperatures, Ultra Violet light and drought.

Brief description of the Figures and Charts

Fig1: Plasmid pCHLSOD construct used as a basic construct for transformation. The arrows named as chl tp and MnSOD indicate the coding regions of chloroplast transit peptide from pea ribulose-1-5-biphosphate carboxylase gene and Nicotiana plaumbaginicolia MnSOD cDNA. The chimeric genes are under the control of 35s promoter and 3' end T7 terminator.

Fig 2: Plasmid pGV2 construct used as a basic construct for co-transformation with pILTAB122. The arrows named as chl tp and MnSOD indicate the coding regions of chloroplast transit peptide from pea ribulose-1-5-biphosphate carboxylase gene and Nicotiana plaumbaginicolia MnSOD cDNA. The chimeric genes are under the control of CsVMV promoter and NOS terminator.

Fig 3: Plasmid pMON1999 construct used as a basic construct for transformation of hygromycin B transferase gnes to get pILTAB122. The construct has CVMV promoter and NOS terminator.

Fig 4: Plasmid pILTAB122 construct used as a basic construct for co-transformation of hygromycin B phosphotransferase along with pGV2 plasmid. The arrows named as hph indicate the coding regions of hygromycin B phosphotransferase. The chimeric genes are under the control of CsVMV promoter and NOS terminator.

Fig 5: Regeneration of putative MnSOD transgenics in japonica rice cultivar TP309. Regenerating calli in shooting media i.e., regeneration media.

Fig 6: PCR amplification analysis of total DNA from chloroplast targeted peptide MnSOD transgenic rice plants. (Plasmid of pILTAB122)

Fig 7: PCR amplification analysis of total DNA from chloroplast targeted peptide MnSOD transgenic rice plants. (plasmid of pGV2)

Fig 8: Southern analysis of chloroplast targeted peptide MnsOD transgenic rice cultivar japonica TP-309.

Fig 9: Northern analysis of the chloroplast targeted peptide MnSOD transgenic plants.

Fig 10: Western blot analysis of untransformed controls and chloroplast targeted peptide MnSOD transgenic plants.

Fig 11: Immunolocalization assay of untransformed control TP-309 and MnSOD transgenic Salween-2.

Fig 12: SOD protein activity assay in untransformed controls and chloroplast targeted peptide MnSOD transgenic rice plants.

Chart 1: Conductance and relative conductance assay of untransformed controls and MnSOD transgenic lines Salween 2 and Godavari 8.

Chart 2: Chlorophyll fluorescence assay of untransformed controls and MnSOD transgenic lines Salween 2 and Godavari 8.

Chart 3: Root length studies with 0μM Menadione in untransformed controls and MnSOD transgenic lines.

Chart 4: Root length studies with 60μM Menadione in untransformed controls and MnSOD transgenic lines.

Chart 5: SOD activity assay in untransformed control TP309 and MnSOD transgenic lines Salween 2 and Godavari 8.

Chart 6: Catalase activity assay in untransformed control TP309 and MnSOD transgenic lines Salween 2 and Godavari 8.

Detailed description of the invention:

The effect of environmental stress on agronomic plants has been a mojor focus on research. Plant productivity is related to the ability of plants to respond to and adapt to environmental stress. Active oxygen species, which are highly toxic to living cells, are produced as byproducts in many bilogical reactions. It has been suggested that active oxygen species are involved in the damage to the plants cells that is caused by environmental stress such as air pollution, high temperature, low water content etc., During stress conditions generation of oxygen radicals is very high, these highly active oxygen radicals(O*) result in causing the following damage to the plant cell:

- Degradation of lipids
- Denaturation of proteins
- Breakdown of Nucleic Acids.

Chloroplasts are potentially the major source of toxic oxygen derivatives in plant tissues. They generate singlet oxygen under high doses of illumination or any other stress conditions. Accumulation of active oxygen species is an unavoidable consequence of photosynthesis, even under the most favourable conditions. To cope with their toxicity, plants have developed a highly efficient anti-oxidant defense system, composed of both enzymatic and non-enzymatic constituents. In plants a number of enzymes involved in free radical scavenging are normally induced in response to a variety of oxidative challenges. However during prolonged stress conditions damage becomes inevitable because the detoxification system becomes saturated. The main players for combating oxidative stress are superoxide dismutase(SOD), ascorbate peroxidases(APX) and catalases. By enhancing the levels of these proteins in transgenic plants, it was attempted to improve the tolerance against oxidative stress. Promising results with transgenic tobacco plants (Bowler et al., 1991; Van Camp et al., 1994) encouraged us to apply this strategy to important crop plants like rice.

Thus, the main aim is to ameliorate the damage caused by hydroxyl radical formed from superoxide radical and hydrogen peroxide. Superoxide dismutase (SOD) is a group of isozymes functioning as superoxide radical scavenger in the living organisms. Thus they protect the plant cells from superoxide radicals. The reaction of SOD is as follows:

$$2H^+ + 2 \cdot O_2^- \longrightarrow H_2O_2 + O_2$$

The produced hydrogen peroxide is then detoxified by catalase or peroxidase.

In eukaryotes, the MnSOD is a nuclear encoded protein that scavenges superoxide radicals in the mitochondrial matrix. By targeting this enzyme to the chloroplast where the generation of superoxide radicals is high during stress conditions, the capacity to scavenge any radical that may be produced can be increased. In an attempt to improve stress tolerance of rice plants, an expression vector containing a *Nicotiana plumbaginicolia* MnSOD cDNA driven by a cauliflower mosaic virus 35S promoter was transferred into the TP309 *japonica* rice callus by particle gun bombardment (using particle accelerator biolistic PDS1000/He). To target this enzyme into the chloroplast, the mature MnSOD coding sequence was fused to a chloroplast transit peptide from Pea ribulose-1-5-biophosphate carboxylase gene.

In particular, the process involves following steps:

Step1: Construction of pGV2 plasmid:

The 1.1 kb chloroplast targeting peptide MnSOD gene was restrict digested from pChltpMnSOD(fig 1) by using BamHI and ClaI. This restrict digested plasmid was cloned into pBSK K-. From this clone, chloroplast targeted peptide MnSOD fragment was restrict digested using KpnI and BamHI restriction enzymes and cloned into pLAU6 (fig2) between CvMv promoter and NOS3' terminator and the resultant plasmid is referred as pGV2 (fig3).

Step2: Construction of pILTAB122:

The 1025 bp hygromycin B phosphotransferase was cloned into pMON1999 vector between 35s promoter and NOS 3' terminator in between KpnI and BamHI and the resultant plasmid is referred as pILTAB122 (fig 4).

The detailed procedure of obtaining transgenic lines and their analysis is listed in the following examples:

Example 1: Transformation of Japonica Rice(TP309) with the above plasmids are as follows.

1. Seeds: TP309 Japonica Rice seeds were provided by the International Laboratory for Tropical Agricultural Biotechnology(ILTAB, La Jolla, USA).

2. Media: NB medium is composed of N6 macro elements (Chu et al.,1975), B5 micro elements (Gamborg et al., 1968), 300 mg/l Casein hydrolysate, 500mg/l L-Proline, 30g/l Sucrose, 2.5 mg/l 2,4-D and 2.5 g/l Phytagel. The NBO medium is NB medium supplemented with 47g/l Mannitol and 47g/l Sorbitol, NH50 medium is NB medium with 50mg/l Hygromycin, PRNH50 medium is NB medium supplemented with BAP 2mg/l, NAA 1mg/l and ABA 5mg/l, RNH50 medium is NB medium supplemented with 3mg/l BAP and 0.5mg/l NAA and ½ MS medium for rooting consists ½ MS salts, ½ B5 Vitamins, Sucrose 10g/l and 2.5g/l Phytagel.

3. Callus induction and selection of regenerable calli: Dehulled mature seeds were surface sterilized in 70% ethanol for 2 min followed by 50% commercial bleach for 30 min. The seeds were then rinsed 3 times with sterilized distilled water. The seeds were placed on pertridishes containing NB medium and incubated at 25'C for 14 days in the dark. At this point the emerging primary calli induced from scutellar region were removed and sub cultured on fresh NB medium plates for another one week at the same conditions. After the subculture many loosely attached small globular calli appeared on top of each compact primary callus, which were gently removed and placed on fresh NB medium plates. The small calli of 1-3mm in diameter were used for transformation.

4. Preparation of Subcultured calli for Bombardment: About 60 embryogenic calli, 2-3mm in diameter were placed at the center of a petridish containing NBO medium. 4 hours after incubation on this medium the calli were immediately subjected to micro-projectile bombardment using the particle accelerator, Biolistic PDS-1000/He (Bio-Rad laboratories,USA).

5. Microprojectile-Mediated transformation: 50μl of freshly prepared 1.8-2.5μM gold particles, 5μg of DNA and 20μl of 0.1M Spermidine were mixed in sequential order. While gently vortexing 50μl of 2.5M CaCl2 was slowly added. The mixture was incubated at room temperature for 10 min and pelleted by spinning in a microfuge for 1 min. The pellet was resuspended in 50μl of 100% cold ethanol, mixed it well by pipetting up and down and distributed 9-10μl samples to microcarrier membranes. The membranes were allowed to dry completely for 5 min and used for transformation immediately. After the transformation the calli were left on same plates and incubated in the dark for 16 hrs.

6. Growth and Selection of Bombarded cells: After 16 hrs the calli were transferred to NH50 plates for selection and incubated at 25'C in the dark for 14 days. After 14 days resistant calli were transferred on to fresh NH50 medium for second selection and incubated at the same conditions for another 14 days. The resistant calli were transferred onto PRNH50 medium for pre-regeneration treatment for one -week. Then the calli transferred onto RNH50 medium for regeneration medium. These plates were kept under a photoperiod of 16 hrs light and 8 hrs dark at 25'C. As plantlets were regenerated they were transferred in to Magenta boxes containing ½ MS medium. When plants were 10cm high they were transferred to Yoshida's culture solution for efficient root development. Two weeks later, the plants were transferred to pots and grown to maturity in the green house.

Example 2: Molecular analysis of the transgenic plants:

1. PCR Analysis of putative Transgenic Plants-DNA samples of the putative transgenic rice plants were extracted from leaf tissues by using standard CTAB method. The DNA concentration was found out by using spectrophotometer. 70ng of DNA was used for PCR reaction. PCR assays were performed in 50μl each reaction with AmpliTaq DNA polymerase. For the presence of Hygromycin Gene, two 20-mers were designed as PCR primers, Forward primer is CVMV End forward: (5'-3') GAT ACA ACT TCA GAG AAA TT, Reverse primer is NOS end reverse: (5'-3') AAA TGT TTG AAC GAT AGG GA. For the presence of MnSOD gene, two 21-mers of plus strand and of minus strand were designed as PCR primers based on the published sequence of the MnSOD gene, Forward primer is SOD 1F(5'-3') CTA CGT CGC CAA CTA CAA CAA, Reverse primer is SOD 1B(5'-3') TAG TCT GGT CTG ACA TTC TTG. 1.1 kb and 850bp of amplified fragments of DNA was expected from transgenic plants for *hph* and MnSOD genes respectively. Ten μl of each reaction was used for electrophoresis in a gel of 1% agarose (Gibco BRL).

2. Southern Analyses of transgenic plants- Genomic DNA was extracted from leaf and stem tissues of rice plants based on CTAB method. 10μg of genomic DNA from each sample, undigested or digested with appropriate restriction endonuclease, was used for electrophoresis in an 1% agarose gel. DNA was blotted to Hybond-N+nylon membrane (Amersham)according to instructions of the manufacturer. Hybridization was carried using the MnSOD probe of 850bp BamH1and Cla1 fragment of pGV2 which was labelled with CDP star Non-radioactive labelling kit(Amersham-UK) based on the manufacturer's instructions.

3. Northern Analysis of transgenic plants-Total RNA was prepared by using Tripure RNA extraction kit (Gibco BRL). 15μg of total RNA was loaded on a formaldehyde denaturing gel, electrophoresed and transferred to nylon membranes according to the manufactures recommendations (Amersham-UK). The MnSOD fragment obtained from parent plasmid pChlSOD, digested with BamH1 and Cla1and eluted by using gel extraction kit was used as probe. Labelling and Hybridisation was carried out according to the Radioisotope labelling kit (Amersham-UK).

Example3: Biochemical and physiological assay:

1. Superoxide dismutase assay- Plant material was homogenised in an equal volume of ice cold extraction buffer (50mM Potassium phosphate,pH 7.8, 0.1%Ascorbate, 0.05% /3-mercaptoethanol, 0.2% Triton X-100), and clarified by centrifugation at 13,000 rpm for 12 minutes. Total protein was measured by protein dye binding assay(Bradford,1976). Samples were separated on non-denaturing 10% polyacrylamide gels and the SOD activity was localised on the gels, using the in situ staining technique of Beauchamp and Fridovich (1971). Inhibitor studies were performed directly on gels in order to distinguish between Cu/Zn,Mn and FeSOD isoforms. These were carried out in the manner described by Sandalio et al.,(1987).

2. Immunolocalisation- The leaf tissues were fixed, embedded in paraffin wax(McFadden et al.,1988) and transverse sections(25mm) were taken using a microtome. The tissue sections were subjected to deparaffining and clearing(Meyerowitz,1987) before probing with antiobodies. Primary antibody: anti-MnSOD(VIB,Ghent,Belgium), Secondary antibody: anti-rabbit IgG conjugated with FITC. The sections were observed under Confocal Microscope (Bio-Rad) at 60X magnification.

3. Growth condtions in stress assessments- Seeds were sterilised with 0.2% $HgCl_2$ for 2 min, rinsed with distilled water and sown on moistened vermiculite. After 5d of germination, the seedlings were transferred to soil:vermiculite mixture(1:2) and watered with nutrient mixture(1/2 Hoglands) for 7 weeks. The plants for the stress tests(SOD and Catalase assays, ESR, PAM and Photosynthesis measurements) were transferred to growth chamber from green house 48 hrs prior to stress tests. This was done so that no additional stress response other than from MV treatment should be present. In the growth chamber the plants were grown in a 16/8 h light/dark cycle at approximately 60% relative humidity, and at day/night temperatures of 25/22'C. The light intenisty was maintained at 200uEm-2s-1 for 12h. Two week old plants were used for Methyl viologen tolerance leaf disc assay, while for all other stress tolerance experiments seven week old plants were used.

4. Methyl viologen tolerance leaf disc assays- Leaf Disc from the third leaf were incubated in the dark at room temperature with an aqueous solution of methyl viologen (MV). The leaves were then incubated for 2h at 30 umol m-2s-1 provided by cool-white fluorescent tubes, and were subsequently dark incubated for another 20 h at 28'C. The MV-dependent oxygen radical damage was estimated from ion leakage out of the discs due to destruction of membrane lipids. Ion leakage was measured as an increase in the conductance of the floating solution.

5. Methyl Viologen treatment- Seven-week-old plants were sprayed with different concentration of MV/Tween 20 solutions (0uM, 5uM, 50uM, 200uM, 500uM and 1000uM). The plants were placed in the growth chamber at 25'C and relative humidity of 60%. The light intensity was maintained at 200uEm-2s-1 for 12h.

6. Chlorophyll Fluorescence- Chlorophyll fluorescence (Schrieber et al., 1986) was measured with a PAM Chl Fluorometer using the emitter-detector unit ED101 (Walz, Effeltrich, Germany). As a measure of the MV-dependent decrease in activity of the reaction centre of PSII,

the variable chlorophyll fluorescence ratio Fv/Fm was used. This ratio gave the excition trapping efficiency when all photochemical traps were open (Gentry et al., 1989). The average of three leaf disc of each MV concentration were used.

7. Assay for MDA by ESR- Each leaf segment (4x20mm) was attached to a open flat cell without a lid, and the cell was inserted to EPR cavity of a JES-RE2X ESR spectrometer (JOEL,Tokyo,Japan).ESR signals from the leaves were recorded at room temperature at a frequency of 9.4GHz close to 337.5mT. Instrument settings were as follows: Microwave power 3mW, receiver gain 2000, time constant 0.3s, modulation amplitude 0.032mT, modulation frequency 100kHz and sweep rate 2.5mT min-1. The leaf segment in the EPR cavity was illuminated with white light from a halogen lamp that was guided to the cell through fiberglass optics. Relative MDA concentrations were obtained as heights of the low field peak of the (first derivative) MDA radical EPR signal.

8. Assay for SOD activity- SOD activity was determined based on the inhibition of the reduction of ferricytochrome c (Cyt C) with O2- generated by the xanthine-xanthine oxidase system (Asada et al.1974), a modification of the method of Fridovich(1969). The amount of xanthine oxidase was adjusted to reduce 14-15nM Cyt c s-1. One unit of SOD was defined as the amount of enzyme that inhibits the reduction of Cyt c by 50%.

9. Cell Viability Assay- Embryonic calli were exposed to Menadione at different concentration i.e., 20mM, 50mM and 100mM. At various time points samples were prepared and staining for viability was done with Trypan Blue.

10. Root Growth Assay- Seeds were grown in the medium containing 0.5% agarose for 7 days, after then the plantlets are transformed into the medium containing 50mM menadione. To generate oxidative stress menadione(2methyl,4napthoquinone) quinone that undergo redox cycling have been widely used to investigate oxidant-induced stress in the cells. Two mechanisms of quinone-induced cytotoxicity have been identified, first quinones are reduced to hydro quinone(Rechheld et al., 1999) so menadione at the concentration of 50 uM used to check the ability of Transgenics to withstand oxidative stress. Observations were taken once in three days up to 16th day.

RESULTS & DISCUSSIONS:

1. Generation of plants, which produce elevated levels of MnSOD:

The entire coding sequence of MnSOD gene was inserted downstream of the CVMV promoter with Chloroplast targeting peptide, followed by Nos terminator. This construct was called as pGV2 (see step 1).

Transgenic TP309-*Japonica* rice plants have been generated by co-transforming pGV2 and pILTAB122 plasmid in the ratio of 3:1. Three-week old scutellum derived callus using particle accelerator Biolistic PDS-1000/He with plasmids pGV2 and pILTAB122. The plasmid pGV2 carried the MnSOD cDNA (Bowler et al.1989) cloned downstream of CvMV promoter and the chloroplast targeting peptide followed by the *nos* terminator. The pILTAB122 carried the

hygromycin B phosphotransferase downstream of 35S CaMV promoter and followed by the *nos* terminator. After culturing regenerates in the presence of Hygromycin, 20 independent lines were obtained (fig 5). The presence of the gene from T_0 to T_2 generations was confirmed by PCR, Southern and Northern and western analysis have been done (Fig 6, 7, 8 7 9 &10). Out of 20 lines in T_0 generation, 17 lines were positive for Hygromycin and 11 lines were positive for Mn SOD (fig 6 & 7). The 11 lines which, were positive for MnSOD and hygromycin, were selected for biochemical and physiological experiments.

2. Results of immunolocalization assays: The production of native engineered protein has been assayed and to determine whether the MnSOD was targeted into correct sub-cellular location, ie., to chloroplast, imunolocalization studies were carried out by incubating the untransformed and transgenic leaves cross sections with polyclonal antibodies raised against *Nicotiana pumbaginifolia* MnSOD. Although expression of the endogenous MnSOD was too low, the exogenous MnSOD was seen (Fig 11).

3. Results of physiological and biochemical assay: To study the cellular effects due to SOD over-production, the use methyl viologen(MV), a herbicide also known as paraquat was used because it is well known that during illumination, MV preferentially accepts electrons from photosystem I, and donates them to oxygen, thus forming the superoxide radical within the chloroplasts. The rectangular leaf discs of equal size, cut from equivalent leaves of untransformed control, transgenic plants were floated on solutions of MV in petri dishes and illuminated as described in examples.

Cellular injury within these leaf discs was assessed firstly measuring the conductance of the floating solution. This measures the leakage of ionic solutes out of the cells and hence gives an indication of membrane damage. Lipid peroxidation resulting from oxidative stress is likely to initiate this membrane deterioration. Light-dependent MV damage as measured by conductance is shown for a control (untransformed TP309) and two chloroplast targeted peptide MnSOD transgenic plants, Salween 2 and Godawari 8. The MnSOD activity was much more pronounced in transgenic lines when compared with the controls (Chart 1). Leaves from transgenic plants were protected against light-dependent MV damage. These leaves showed less damage than the controls. There were differences between the performances of the two transgenic lines; it could be due to more copy no of the MnSOD gene in Godawari 8 (11 copies) and Salween 2 (5 copies) (Fig 8). It was further observed that the effects of MnSOD overproduction is consistent with the fact that the formation of superoxide radicals is being biased to the chloroplasts in light conditions than in the mitochondria.

To examine chloroplast-localized effects, we have used chlorophyll fluorescence techniques to determine the integrity of various components of the photosynthetic apparatus (for review, see Krause and Weis, 1984). The parameters studied were the quantum yield of exciton trapping by the reaction center of photosystem II will be documented. The parameter gives a measure of the integrity of the photosystem II reaction center. From this documentation it can revealed that there was more damage in the photosystem II in controls, less damage in Salween 2 transgenic plants when compared to controls, but there was integrity of photosystem II without damage significantly when compared to controls (**Chart:2**) Hence high level of MnSOD

overproduction in the chloroplasts can protect both the photosynthetic apparatus and the cellular membranes from the MV-generated damage.

4. Oxidative stress inhibits the cell division in the root meristem via specific cell cycle arrest at G1 and G2 phases (comparison shown between transgenic and non transgenic):

To know the growth pattern of the roots under the oxidative stress conditions between transgenic over-expressing MnSOD and control plants, Root growth measurements were taken by growing plants with the medium containing 50uM menadiaone. Oxidative stress is induced by the chemical Menadione (2 methly 4-napthoquinone). Menadione that, under go redox cycling has been widely used to investigate oxidative induced stress in the cells. Menadione may undergo either one or two electron reduction. The one electron reduction of the quinone forms a semiquinone radical; this process catalyzed by a variety of flavoenzymes, including NADPH-cytochrome p-450 reductase, NADPH-ubiquinone oxidoreductase. In the presence of di-oxygen, the semiquinone radical can be deoxidized to the parent quionone with concomitant formation of superoxide anion, O_2^-. The enzymatic or spontaneous dismutation of O_2^- yields O_2 and H_2O_2 which in presence of certain metals, can react with O_2^- to form more reactive oxygen species such as the hydroxyl radical(OH) and singlet oxygen($1O_2$). The electron reduction of the certain quionone to the corresponding hydroquionone is catalyzed by the flavoenzymes without formation of semiquionone free radical intermediate (Michael et al., 1994: Donato et al., 1984). Observations were taken once in three days till the 16th day under sterile conditions using 30cm scale. From the (**chart 3**-with out menadione treatment & 4-with 50μm menadione treatment) it is clearly observed that control and transgenic plants were growing well in the absence of menadione but in case of treatment of 50μM menadione the root length decreased considerable on 7th day in controls, but in case of transgenic Godawari 8, salween 2 etc., showed better root growth till the end of the 16th day. This data strongly suggests that oxidative stress inhibit cell division in the root meristem via specific cell cycle arrest at G1 and G2 phases. This observation suggests an adaptive behaviour of the plants to oxidative stress is very quick in SOD over expressing, as evident in transgenic lines Godavari 8 and Salween 2.

5. ***Increase in catalase activity due to the over-expression of SOD activity:***

It has been shown that the catalase activity is increased due to the over-expression of MnSOD, this is because superoxide radicals are ubiquitously generated in many biological oxidations in various compartments of the cell. The toxicity of superoxide radicals has been attributed to their interaction with other cellular constituents, in particular with hydrogen peroxide. In the presence of trace amounts of iron salts, the combination of superoxide radicals and hydrogen peroxide leads rapidly to the formation of highly reactive hydroxyl radicals (OH-). The excess of Hydrogen peroxide is metabolized by catalase activity leading into water and free ozygen. The observations seen in this experiment, as the SOD activity **(Chart 5)** increases the catalase activity in transgenic plants was increased when compared to controls **(Chart 6).**

In summary, we have shown that, where MV damage is light-dependent, high level overproduction of MnSOD in the chloroplasts can significantly reduce the normal level of oxidative damage, protecting both the compartment where free radicals were generated and the

cellular membranes as well. The experiments described above have been repeated with three sets of plants but in all cases the average is taken for consideration.

Also, it is clear from the results that SOD can be manipulated to give resistance to oxidative stress although there is clearly a fine line between benefit and injury, dependent upon the level of overproduction, the type of SOD used and the endogenous scavenging systems of the organism, with everything being ultimately dependent upon the $O_2^{\cdot}$:H_2O_2 ratio. Our results support the use of MnSOD expressed at high levels are the most promising route of SOD for developing stress tolerance in plants. Finally it remains to be seen whether visible phenotypes will be observed in the whole plant even with regard to economically relevant stress conditions such as chilling, high light intensitites, drought, pollutants such as ozone and UV etc. However, the degree of protection observed at the cellular level will be sufficient to provide such a resistant phenotype at the whole plant.

We claim:

1. A method for producing a transgenic indica rice varieties comprising:

   (a) Constructing an expression vector for plant transformation that comprises a promoter, a superoxide dismutase (SOD) gene derived from *Nicotiana plumbaginicolia L.*, and a transit peptide;

   (b) Transferring the vector constructed in step (a) to a transformant;

   (c) Co-culturing the transformant of step (b) with the plant tissue; and

   (d) Regenerating the transformed tissue into a mature transgenic plant.

2. A claim as in claim 1, wherein, the said transit peptide is a Pea ribulose-1-5-biphosphate carboxylase gene.

3. A claim as in claim 1, wherein, the said promoter is a CvMV promoter.

4. A claim as in claims 1 & 4, wherein, the terminator used is the NOS terminator.

5. A method of conferring increased tolerance to environmental stress in the rice plants, by transforming cells of the said plant with second DNA sequence encoding a transit peptide to facilitate the transportation of said MnSOD gene directed to a plant cell organelle.

6. A claim as in claim 5, wherein, the said plant cell organelle is a chloroplast.

7. A transgenic rice variety, that produces high levels of superoxide dismutase (SOD).

8. A transgenic rice variety specifically, Godavari 8 and Salween 2., that produces high levels of superoxide dismutase (SOD).

9. A claim as in claims 1, 5 & 8, wherein, the transgenic plants confer increased tolerance to environmental stress conditions such as drought, salinity, ultra violet radiation, heat and cold.

10. A claim as in claims 1, 5 & 8, wherein, the transgenic plants confer increased yield under environmental stress conditions.

11. A claim as in claims 1, 5 & 8, wherein, the transgenic plants confer increased tolerance to pathogen attack.

12. A claim as in claims 1, 5 & 8, wherein, the transgenic plants will play an important role in the food industry by increasing the shelf life.

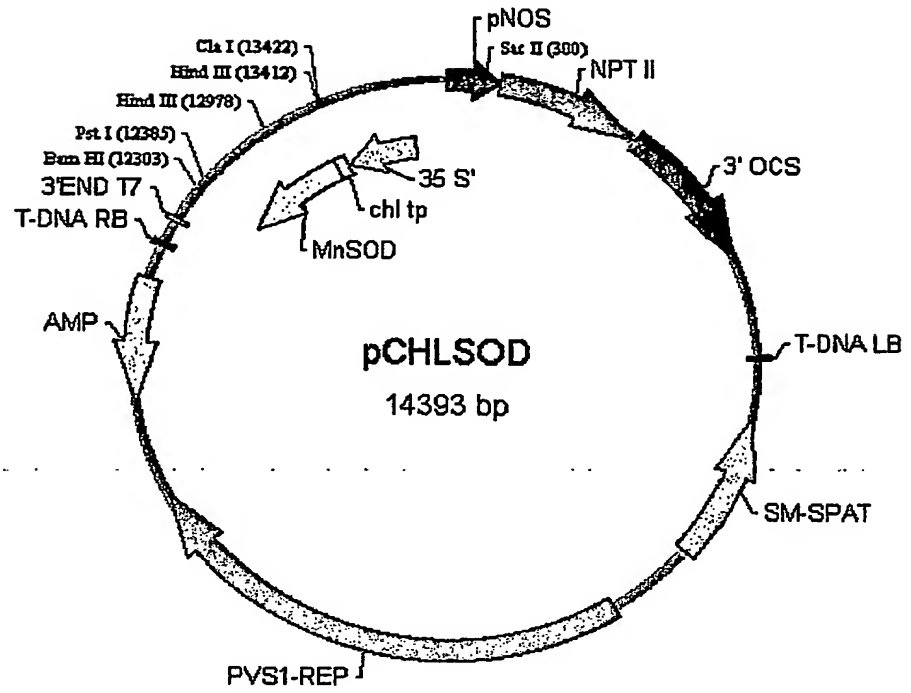


Fig1: Plasmid pCHLSOD construct used as a basic construct for transformation. The arrows named as chl tp and MnSOD indicate the coding regions of chloroplast transit peptide from pea ribulose-1-5-biphosphate carboxylase gene and Nicotiana plaumbaginicolia MnSOD cDNA. The chimeric genes are under the control of 35s promoter and 3' end T7 terminator.

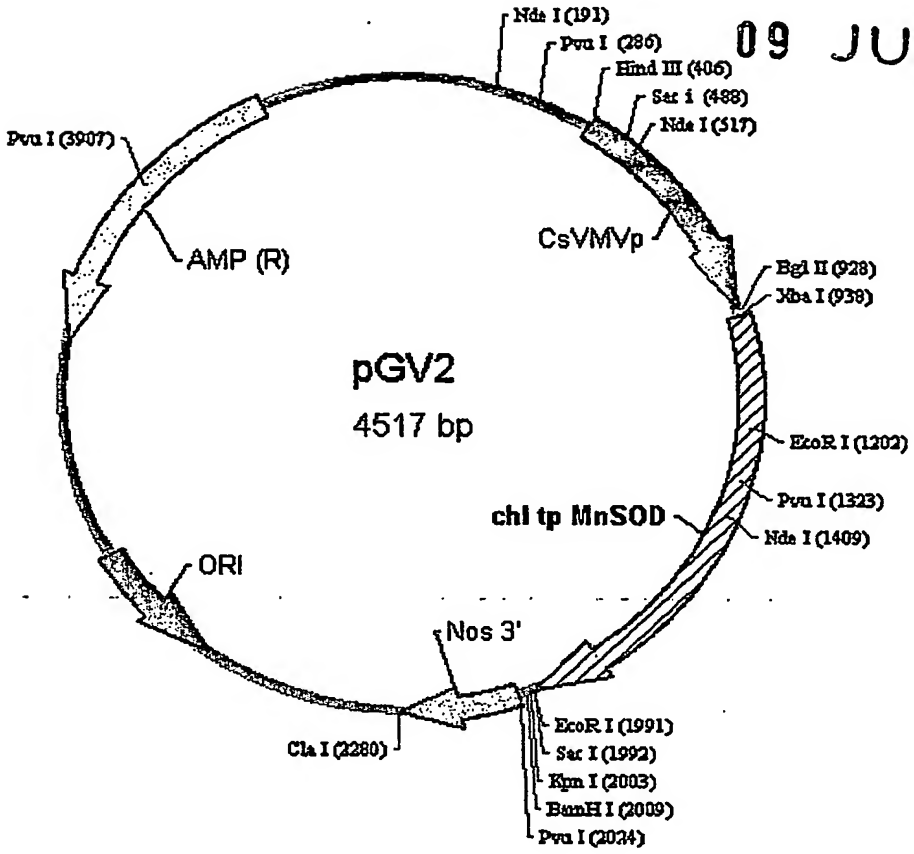


Fig 2: Plasmid pGV2 construct used as a basic construct for co-transformation with pILTAB122. The arrows named as chl tp and MnSOD indicate the coding regions of chloroplast transit peptide from pea ribulose-1-5-biphosphate carboxylase gene and Nicotiana plaumbaginicolia MnSOD cDNA. The chimeric genes are under the control of CsVMV promoter and NOS terminator.

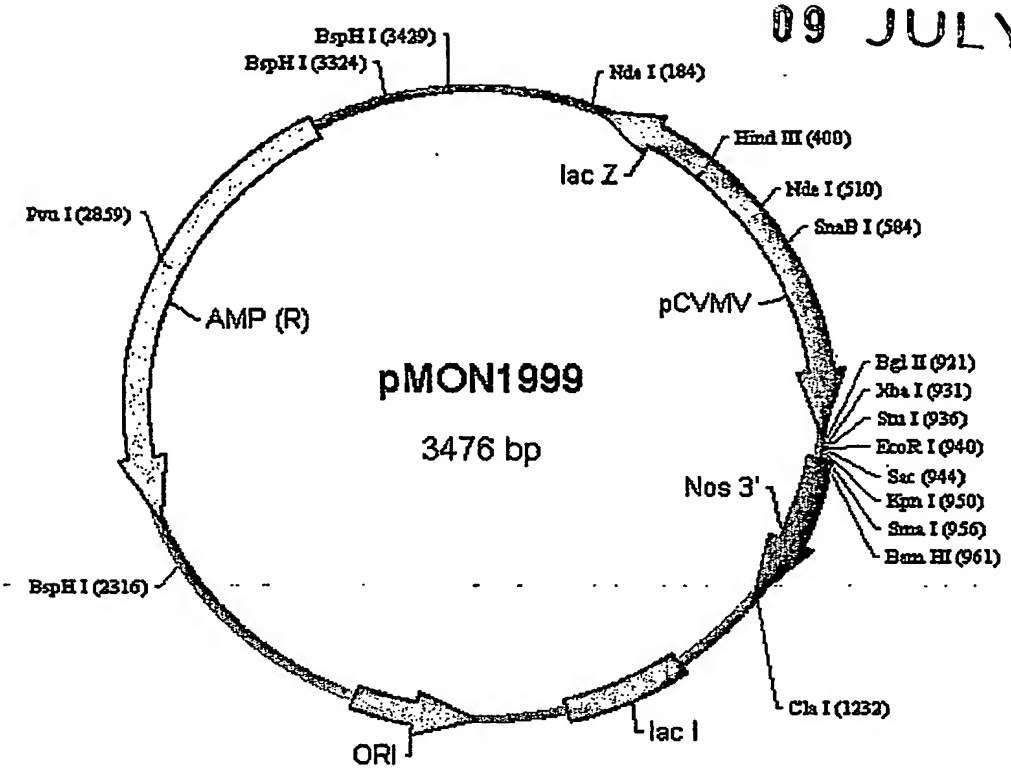


Fig 3: Plasmid pMON1999 construct used as a basic construct for transformation of hygromycin B transferase gnes to get pILTAB122. The construct has CVMV promoter and NOS terminator.

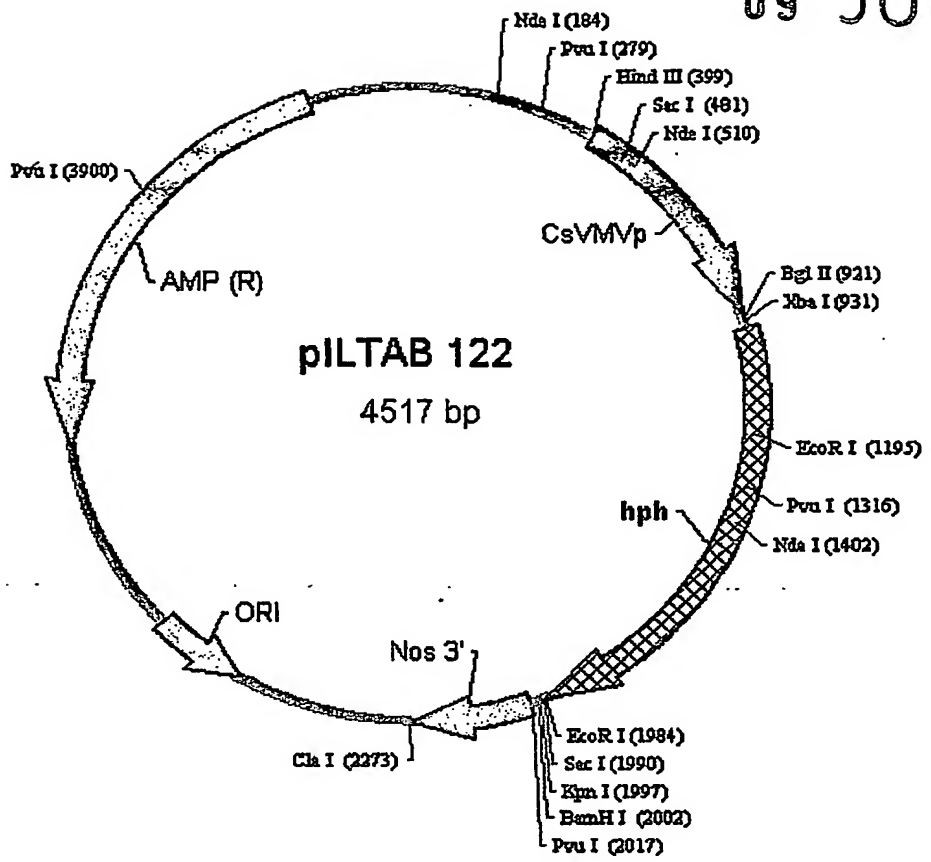


Fig 4: Plasmid pILTAB122 construct used as a basic construct for co-transformation of hygromycin B phosphotransferase along with pGV2 plasmid. The arrows named as hph indicate the coding regions of hygromycin B phosphotransferase. The chimeric genes are under the control of CsVMV promoter and NOS terminator.

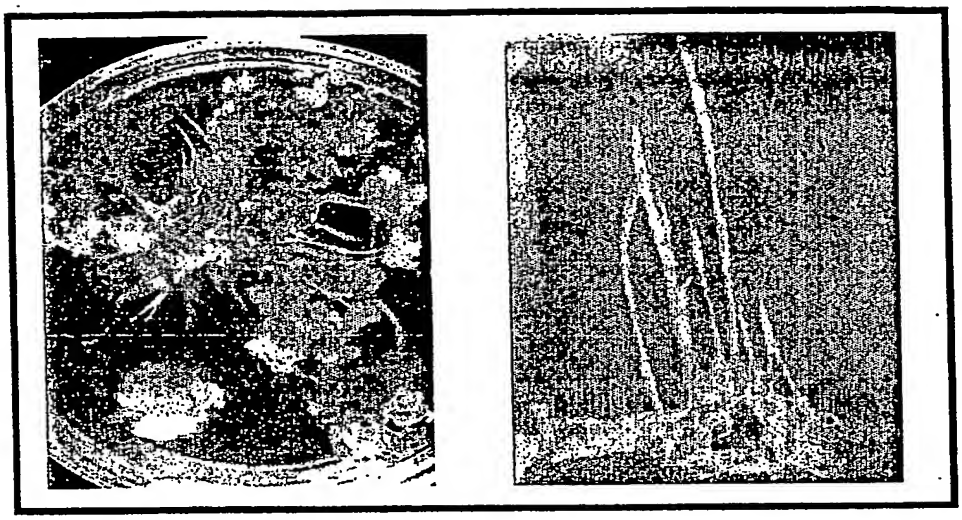


Fig 5: Regeneration of putative MnSOD transgenics in japonica rice cultivar TP309. Regenerating calli in shooting media i.e., regeneration media

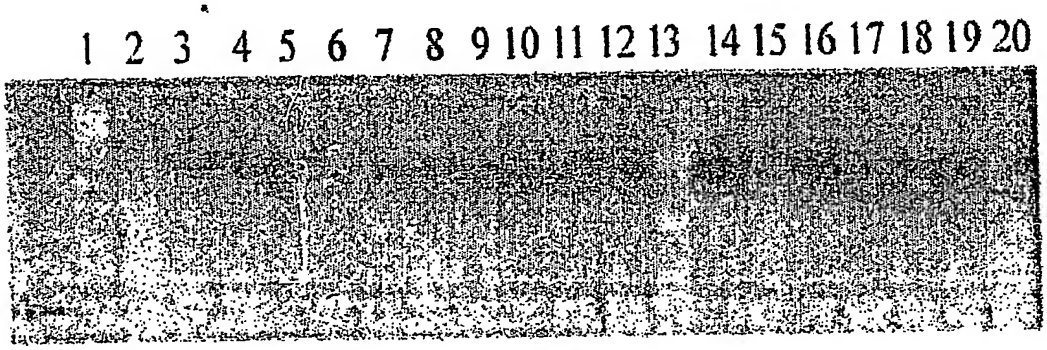


Fig 6: PCR amplification analysis of total DNA from chloroplast targeted peptide MnSOD transgenic rice plants. Total DNA from the rice plants was prepared as described and subjected to PCR amplification with amplification primers located at the start of start and end of hygromycin B phosphotransferase gene. Lanes 3-20: DNA from plants of different transgenic lines, 1: 1 Kb ladder, and 22: positive controls (Plasmid of pILTAB122)

Note: All the plants from 3-20 except 7, 9& 13 showed clear amplification and hence presence of transgene

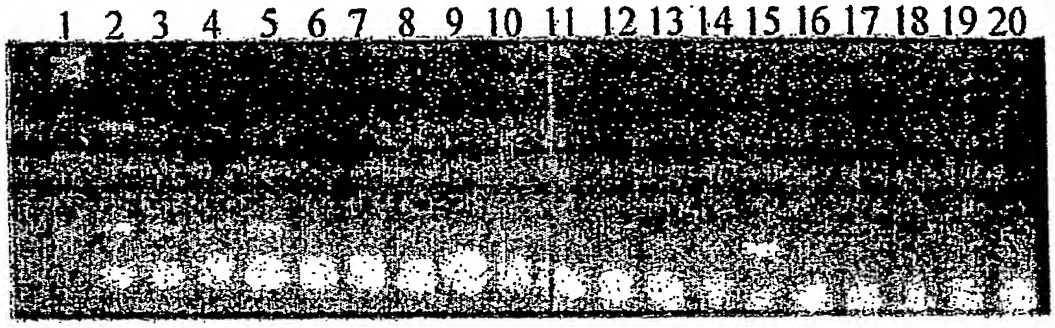


Fig 7: PCR amplification analysis of total DNA from chloroplast targeted peptide MnSOD transgenic rice plants. Total DNA from the rice plants was prepared as described and subjected to PCR amplification with amplification primers located at the start of start of Chloroplast targeted peptide and end of MnSOD gene. Lanes 3-20: DNA from plants of different transgenic lines, 1: 1 Kb ladder, and 2: positive controls (plasmid of pGV2)

Note: The plants from 3, 4, 5, 7, 8, 9, 10, 11, 13, 14 and 15 showed clear amplification and hence presence of transgene (both hygromycin B phosphotransferase gene and MnSOD gene)

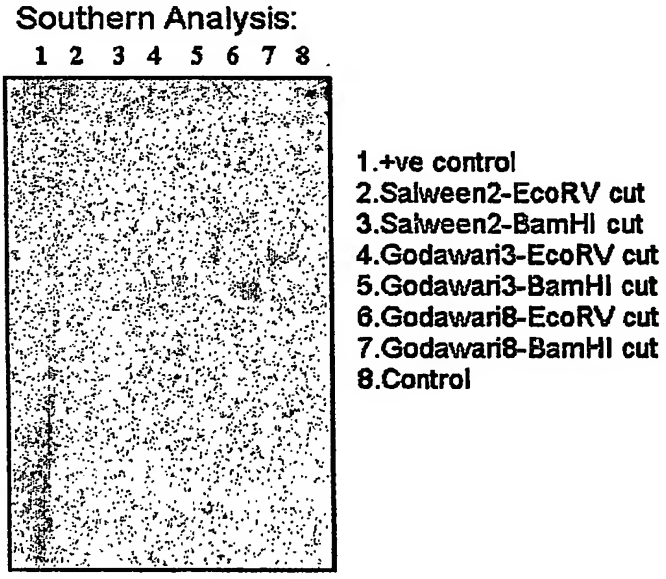


Fig 8: Southern analysis of chloroplast targeted peptide MnsOD transgenic rice cultivar japonica TP-309. The genomic DNA was extracted according to standard CTAB protocol and Southern analysis was done according to standard operating protocol as described. Lane 1- positive control; Lane 2- MnSOD transgenic plant Salween 2 restrict digested with EcoRV; Lane 3- MnSOD transgenic plant Salween 2 restrict digested with BamHI; Lane 4- MnSOD transgenic plant Godavari 3 restrict digested with EcoRV; Lane 5- MnSOD

transgenic plant Godavari 3 restrict digested with BamHI; Lane 6- MnSOD transgenic plant Godavari 8 restrict digested with EcoRV; Lane 7- MnSOD transgenic plant Godavari 8 restrict digested with BamHI and lane 8 negative control.

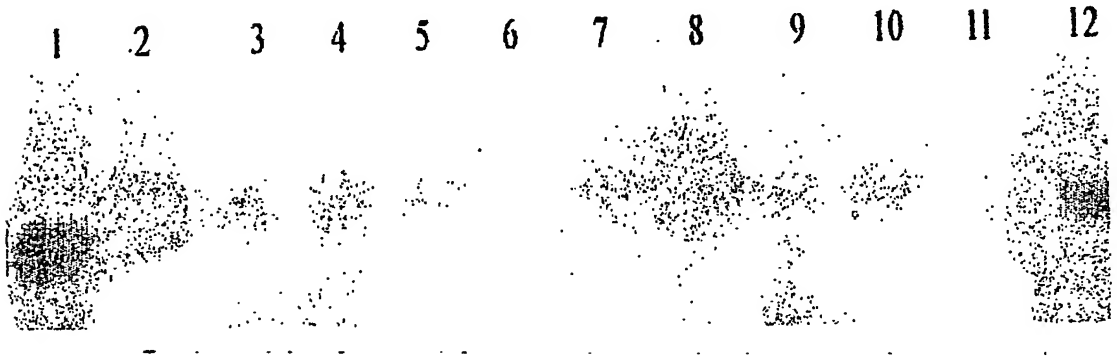


Fig 9: Northern analysis of the chloroplast targeted peptide MnSOD transgenic plants. RNA was extracted using standard procedure (Sambrook et al.,). Lanes 1- positive control; 2- Ganga 12; 3- Godavari 3; 4- Godavari 8; 5- Cauvery 8; 6- Chari 13; 7- Darling 2; 8- Orange 8; 9- Cooper 1; 11- Salween 2 and 12- Salween 4.

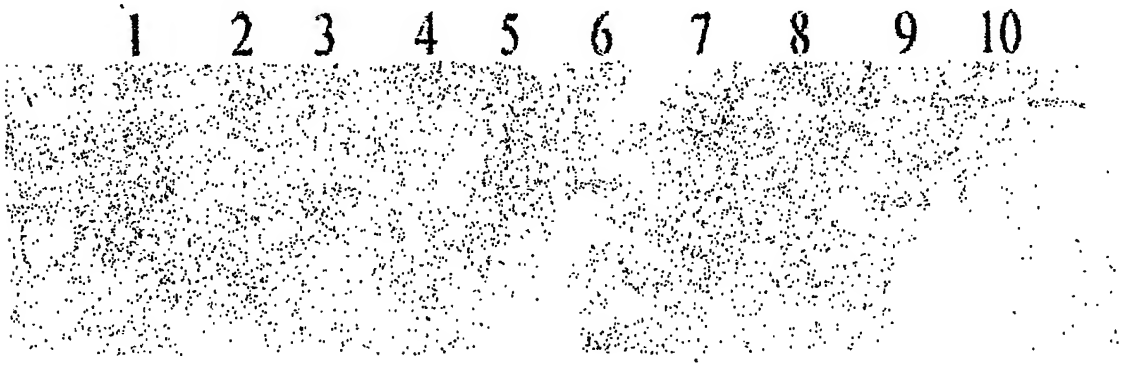


Fig 10: Western blot analysis of untransformed controls and chloroplast targeted peptide MnSOD transgenic plants. Lane 1- protein marker; 2- untransformed control plant; 3- Cauvery 7; 4- Chary 8; 5- Ganga 12; 6- Godavari 8; 7- Hwang HO 2; 8- Orange 2; 9- Salween 2 and 10- orange 6.

Transgenic plants Cauvery 7, Ganga 12, Godavari 8 and Salween 2 showed increased protein levels of MnSOD.

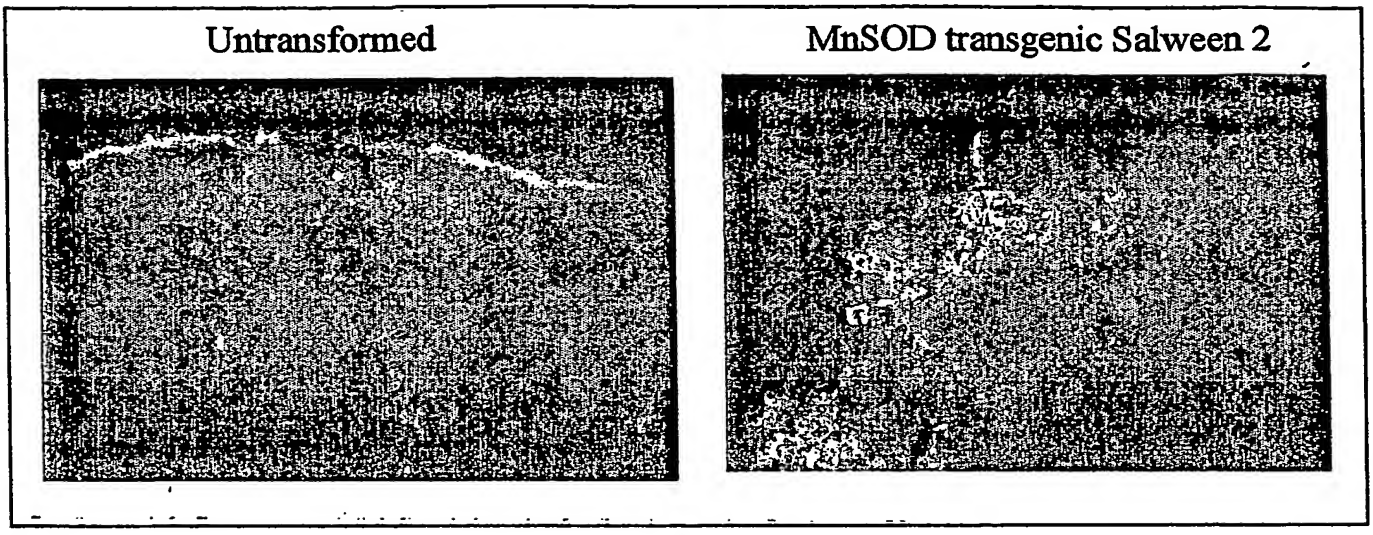


Fig 11: Immunolocalization assay of untransformed control TP-309 and MnSOD transgenic Salween-2. Immunolocalization was performed according to standard protocol as described.The exogeneous MnSOD production was found to be more in MnSOD transgenic plants.

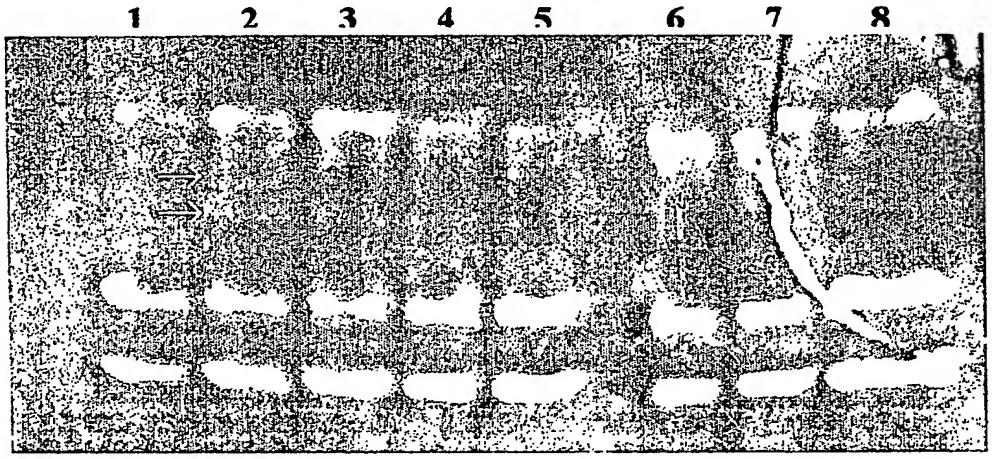
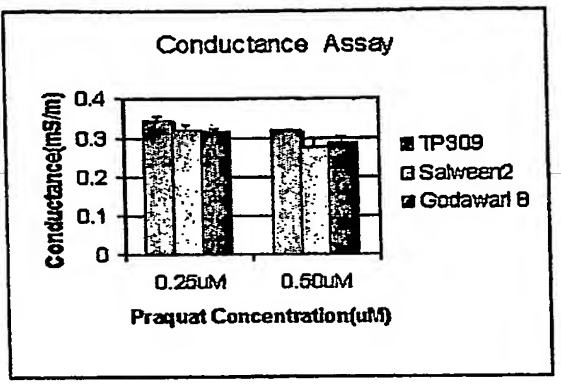
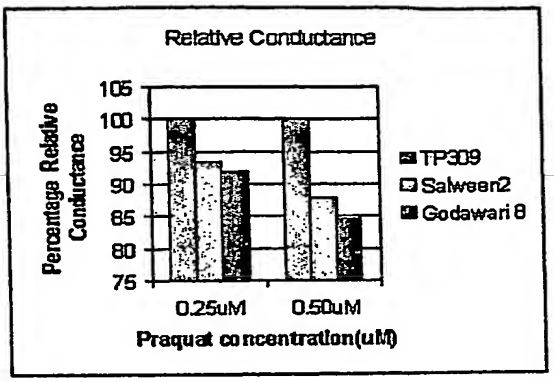


Fig 12: SOD protein activity assay in untransformed controls and chloroplast targeted peptide MnSOD transgenic rice plants. Lane 1- untransformed controls; 2- Ganga 12; 3- Ganga 13; 4- Godavari 3; 5- Chari 8; 6- Chari 10; 7- Chari 13 and 8- Salween 2.

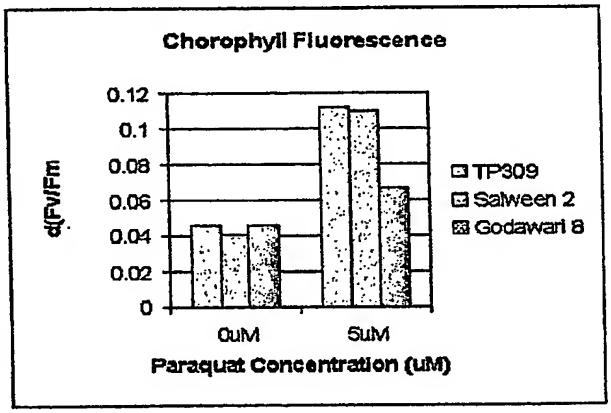
All the transgenic plants showed elevated levels of SOD protein activity than controls





Electrolyte leakage from Paraquat- treated leaf discs conductivity values at zero hours were subtracted from those at 22 hours after the beginning of illumination and relative values of the differences at 0.25 and 0.50 uM Paraquat were determined. The mean values + SE obtained from three experiments are shown. The value of 100% relative conductivity corresponds to 0.344 mS/m (mean ±SE, n=8).

Chart 1: Conductance and relative conductance assay of untransformed controls and MnSOD transgenic lines Salween 2 and Godavari 8. The assay was done according to the standard protocol as described.



Methylviologen effects on chlorophyll fluorescence:(Fv/Fm) ratio represents degree of chlorophyll protection. d(Fv/Fm) ratio represents difference between before and after Methylviologen treatment.

Chart 2: Chlorophyll fluorescence assay of untransformed controls and MnSOD transgenic lines Salween 2 and Godavari 8. The assay was done according to the standard protocol as described.

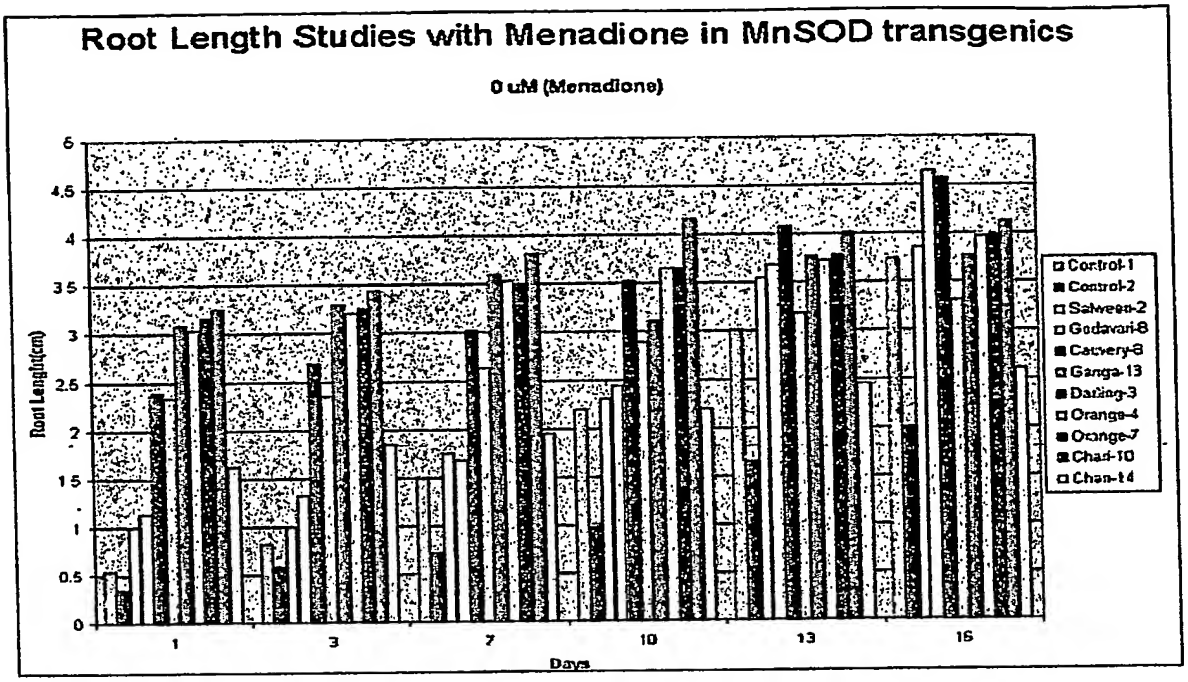


Chart 3: Root length studies with menadione in untransformed controls and MnSOD transgenic lines. The assay was done at different dates of seedling growth ranging from 0 days to 16 days. Transgenic lines showed more root growth than the untransformed control plants even at 0μM menadione concentration.

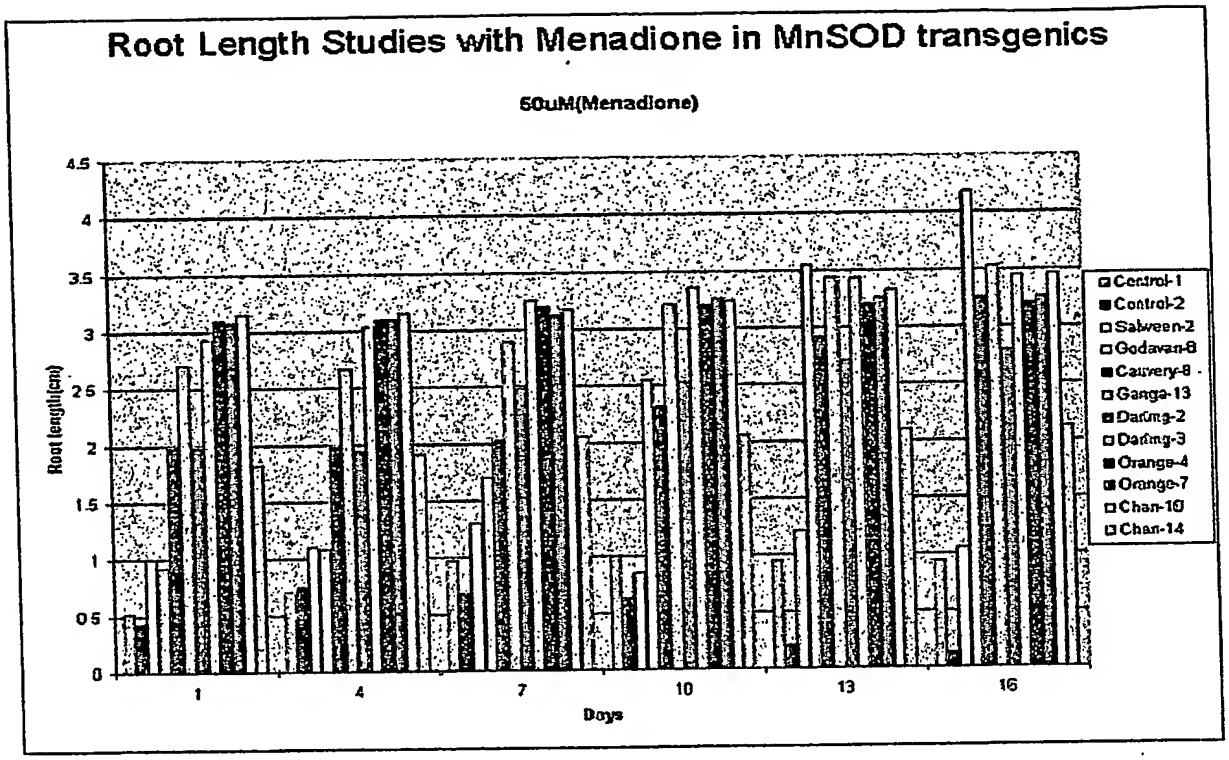
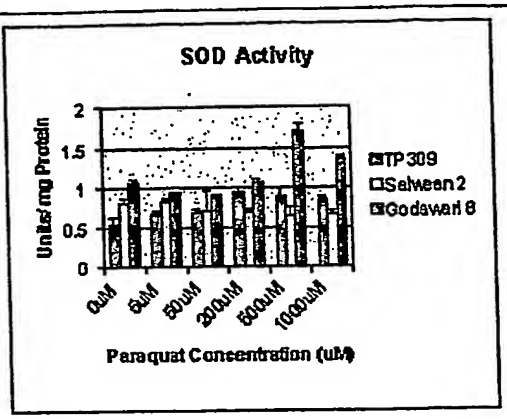
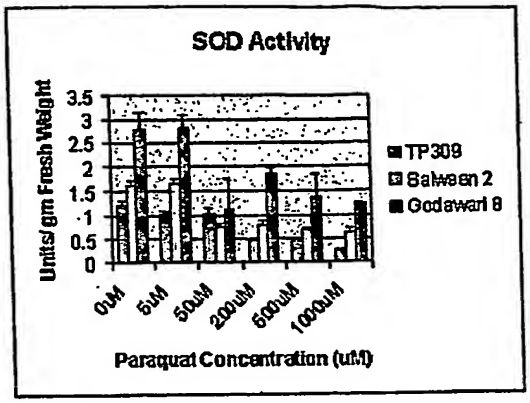


Chart 4: Root length studies with menadione in untransformed controls and MnSOD transgenic lines. The assay was done at different dates of seedling growth ranging from 0 days to 16 days at various concentration of menadione. Transgenic lines showed better perfomance than controls with respect to root growth.

AMENDED SHEET

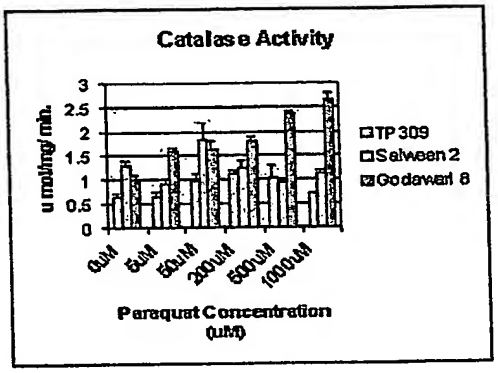


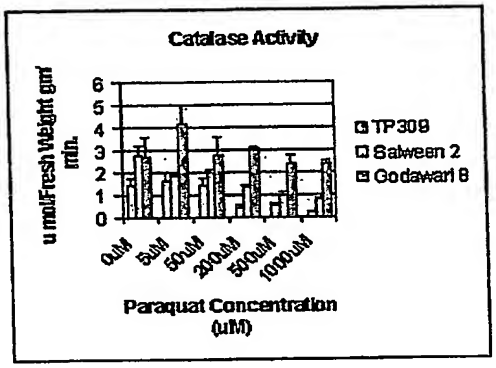


FREE RADICAL SCAVANGING ENZYME :SOD ACTIVITY

$$O_2^- + O_2^- + 2\,H^+ \text{---------} > H_2O_2 + O_2$$

Chart 5: SOD activity assay in untransformed control TP309 and MnSOD transgenic lines Salween 2 and Godavari 8. The assay was done according to the standard protocol as described.





HYDROGEN PEROXIDE METABOLISM BY CATALASE

$$H_2O_2 + H_2O_2 \text{----------} > 2H_2O + O_2$$

Chart 6: Catalase activity assay in untransformed control TP309 and MnSOD transgenic lines Salween 2 and Godavari 8. The assay was done according to the standard protocol as described.